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Comparison of the Roles of Calcineurin in Physiology and Virulence in Serotype D and Serotype A Strains of *Cryptococcus neoformans*

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The calcineurin gene was cloned and disrupted in serotype D strains of *Cryptococcus neoformans*. Serotype A and serotype D calcineurin mutants were inviable at 37°C and avirulent in mice, whereas only serotype A mutants were cation stress sensitive. Thus, calcineurin plays conserved and divergent roles in serotype A and serotype D strains.

Cryptococcus neoformans is an encapsulated basidiomycete that is the most common cause of systemic mycosis in AIDS patients. *C. neoformans* strains are classified into five serotypes (A, B, C, D, and AD) and two varieties: *C. neoformans* var. *neoformans* (serotypes A, D, and AD) and *C. neoformans* var. *gattii* (serotypes B and C). Serotype A and serotype D strains exhibit significant variation and may represent distinct varieties that have diverged in ~18 million years of evolution (8, 9, 16, 19, 25). *C. neoformans* virulence factors include the capsule (3–5), melanin (22), prototrophy (17), and growth at 37°C (14). The protein phosphatase calcineurin is required for *C. neoformans* growth at 37°C and virulence (14).

Calcineurin is a Ca^{2+}-calmodulin-activated phosphatase with catalytic and regulatory subunits (10). Calcineurin is the target of the T-cell-specific immunosuppressants cyclosporine (CsA) and tacrolimus (FK506). CsA and FK506 suppress the immune system by inhibiting calcineurin and preventing gene expression during T-cell activation. The antifungal activities of CsA and FK506 are mediated by a similar mechanism involving fungal homologs of calcineurin and the drug-binding proteins cyclophilin A and FKBP12 (1, 2, 6, 7, 14, 15).

Calcineurin has been identified from several fungi and regulates cell cycle progression in *Aspergillus nidulans* (21), hyphal elongation and growth in *Neurospora crassa* (11, 20), and mating and cytokinesis in *Schizosaccharomyces pombe* (18, 28). In *Saccharomyces cerevisiae*, calcineurin is required for recovery from pheromone arrest (12, 27) and regulates cation homeostasis and cell wall biosynthesis via the transcription factor Crz1 (1, 13, 23, 24).

The calcineurin gene has been identified, sequenced, and disrupted by homologous recombination in *C. neoformans* serotype A strain H99 (14). Calcineurin is essential for growth at 37°C, virulence in a rabbit model of cryptococcal meningitis, and cation homeostasis (14). Here, we isolated and disrupted the gene encoding the calcineurin A catalytic subunit (*CNA1*) from the congenic serotype D strains of *C. neoformans* and compared the functions of calcineurin in serotype A and serotype D strains.

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Identification, sequence, and disruption of the serotype D calcineurin A *CNA1* gene. The *CNA1* gene encoding calcineurin A was cloned from the serotype D strain JEC21 by PCR amplification with primers to conserved sequences in the serotype A *CNA1* gene. A 1.8-kb *CNA1* gene fragment was sequenced, revealing identity to calcineurin genes, and used in Southern blot analysis to show that the *CNA1* gene is contained on an 8-kb *Eag*I fragment. The gene was recovered from a size-selected *Eag*I genomic library and sequenced. There were seven amino acid differences between the calcineurin A protein in serotype A and serotype D.

To disrupt the serotype D *CNA1* gene, the *ADE2* gene was inserted at an *Hpa*I site in the *CNA1* gene. The *cna1*::*ADE2* allele was transformed with a biolistic apparatus into serotype D *MAT*α *ade2* strain JEC50 and *MAT***a** *ade2 ura5* strain JEC156. A total of 3 of 200 Ade^+ transformants (1.5%) were viable at 24°C, grew poorly at 30°C, and were inviable at 37°C. The *cna1* mutation confers a more severe growth defect at 30°C in serotype D than in serotype A strains, consistent with findings that FK506 and CsA are more toxic at 30°C to serotype D than to serotype A strains (14). Southern blotting confirmed that the *CNA1* gene was replaced by the *cna1*::*ADE2* allele without ectopic integration in all three isolates (data not shown). In one isolate, the wild-type *CNA1* gene was precisely replaced by the *cna1*::*ADE2* allele by a double crossover. In two other isolates, tandem integrations had occurred at the *CNA1* locus. By an overlay blot with ^{125}I-calmodulin, calcineurin was expressed in *CNA1* wild-type strains but not in the *cna1*::*ADE2* mutant strains (data not shown). Genetic crosses and analysis of basidiospores by micromanipulation revealed that the *ADE2* gene was integrated into a single genomic locus. All Ade^+ meiotic segregants exhibited the *cna1* temperature-sensitive growth defect, and all Ade^- segregants grew at 37°C, indicating that the *cna1* mutation is linked to the temperature-sensitive defect and the *ADE2* gene.

Comparison of calcineurin *cna1* mutant phenotypes in serotype A and serotype D strains. The growth of serotype A and serotype D wild-type and *cna1* mutant strains was compared using a quantitative dilution assay for viability at 37°C and sensitivity to Na^+ and Li^+ (Fig. 1). At 25°C, growth of serotype A and serotype D *cna1* mutants was comparable to that of wild-type strains. At 30°C, viability of serotype D *cna1* mutants was severely reduced compared to the wild-type strain; growth of wild-type and *cna1* mutant serotype A strains did not differ

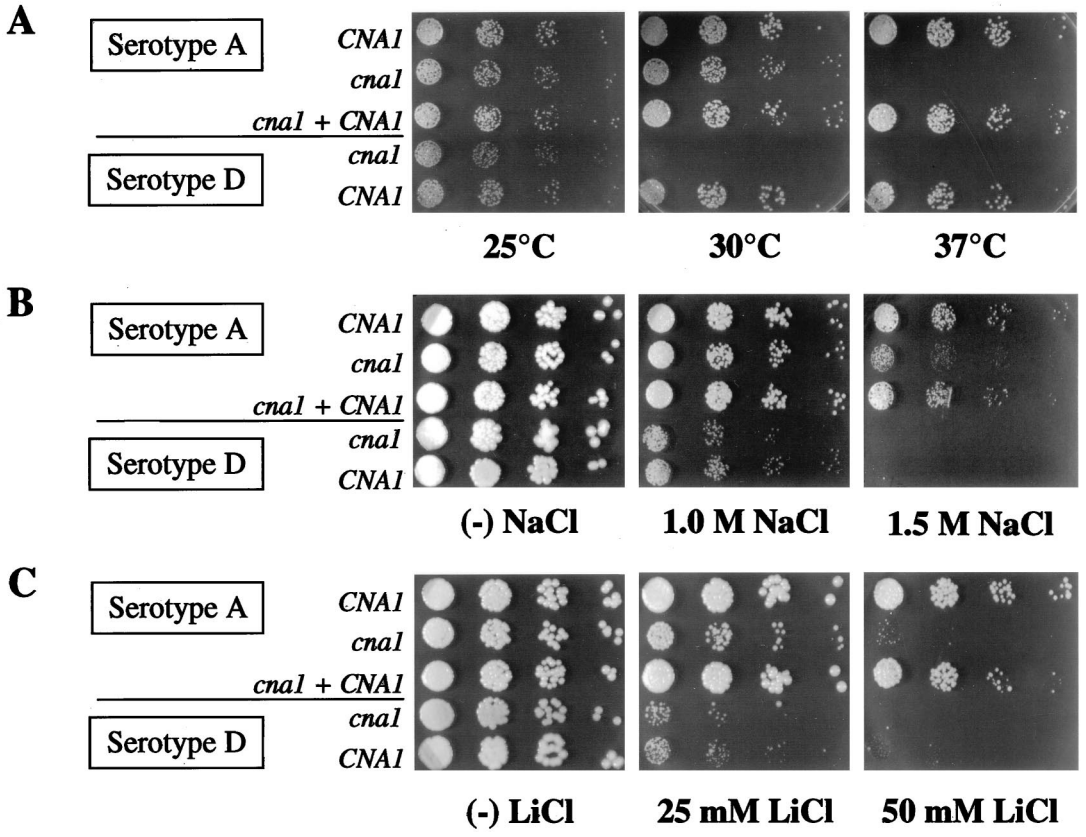

FIG. 1. Growth properties of serotype A and serotype D calcineurin mutants of *C. neoformans*. Isogenic serotype A *CNA1* wild-type (H99), calcineurin A *cna1* mutant (AO4), and *cna1* plus *CNA1* reconstituted (AO10) strains and isogenic serotype D *CNA1* wild-type (JEC21) and *cna1* mutant (MCC3) strains were grown in YPD liquid medium and spotted onto YPD medium to compare growth at different temperatures (25, 30, and 37°C) (A), on medium containing 0 (−), 1, or 1.5 M NaCl (B) and 0 (−), 25, or 50 mM LiCl (C). Each dilution contained (from left to right) 1,250, 250, 50, and 10 cells of each strain. Cells shown in panels B and C were incubated for 72 h at 25°C.

at 30°C. At 37°C, both serotype A and serotype D *cna1* mutant strains were inviable. Reintroduction of the *CNA1* gene restored growth at 37°C; thus, calcineurin is required for growth at 37°C for both serotype A and serotype D strains.

Growth of serotype A *cna1* mutants was impaired compared to that of the *CNA1* wild-type or *cna1* plus *CNA1* strains on 1.5 M NaCl (Fig. 1B). Serotype D *cna1* mutants showed only a slight reduction in the number of CFU on 1 M NaCl. The growth of wild-type serotype D strains was completely inhibited on 1.5 M NaCl, whereas serotype A wild-type strain H99 was viable (Fig. 1B). Thus, the congenic serotype D strain are more sensitive to cation stress than serotype A strain H99, and the *cna1* mutation has little effect on cation resistance in serotype D.

Growth of serotype A *cna1* mutants was compromised on yeast extract-peptone-dextrose (YPD) medium with 50 mM LiCl compared to the wild-type or *cna1* plus *CNA1* reconstituted strains (Fig. 1C). Growth of serotype D *cna1* mutants was not reduced on 25 mM LiCl compared to wild-type strains. Both the wild-type and the *cna1* mutant serotype D strains were inviable on 50 mM LiCl, in contrast to serotype A strain H99 (Fig. 1C).

Calcineurin is required for virulence of serotype A and serotype D strains in mice. We tested if calcineurin is required for virulence of serotype D strains of *C. neoformans* in the murine model of cryptococcosis. Each animal was infected with 10^7 *C. neoformans* cells by lateral tail vein injection. Ten animals were analyzed for each strain, and survival was monitored as the endpoint.

Infection of BALB/c mice with the serotype D *CNA1* wild-type strain JEC21 resulted in the death of 50% of infected animals by day 153 and 80% by day 195. In comparison, all 10 mice infected with the isogenic *cna1* mutant strain MCC2 survived to day 238 (Fig. 2A). No viable fungal cells could be cultured from the brains or lungs of mice infected with the serotype D *cna1* mutant strain, indicating that the mutant had been eradicated (results not shown).

Comparable studies were conducted with the serotype A *CNA1* wild-type, *cna1* mutant, and *cna1* plus *CNA1* reconstituted strains (Fig. 1B). Infection with *CNA1* wild-type strain H99 resulted in 50% mortality by day 8 and 100% mortality by day 20. The *cna1* mutation severely attenuated virulence, and all 10 mice infected with the *cna1* mutant survived to day 100. Reintroduction of the wild-type *CNA1* gene restored the virulence of the *cna1* mutant, albeit not to the wild-type level, resulting in 100% mortality by day 38. Thus, calcineurin is required for virulence of serotype A strain H99 in two different animal models.

Studies conducted with C5-deficient mice resulted in 100 and 70% mortality at day 120 following infection with wild-type strains JEC20 or JEC21, whereas 100% of mice infected with serotype D *MAT***a** or *MAT*α *cna1* mutant strains survived to day 120 (Fig. 2C and D). Thus, calcineurin is required for the

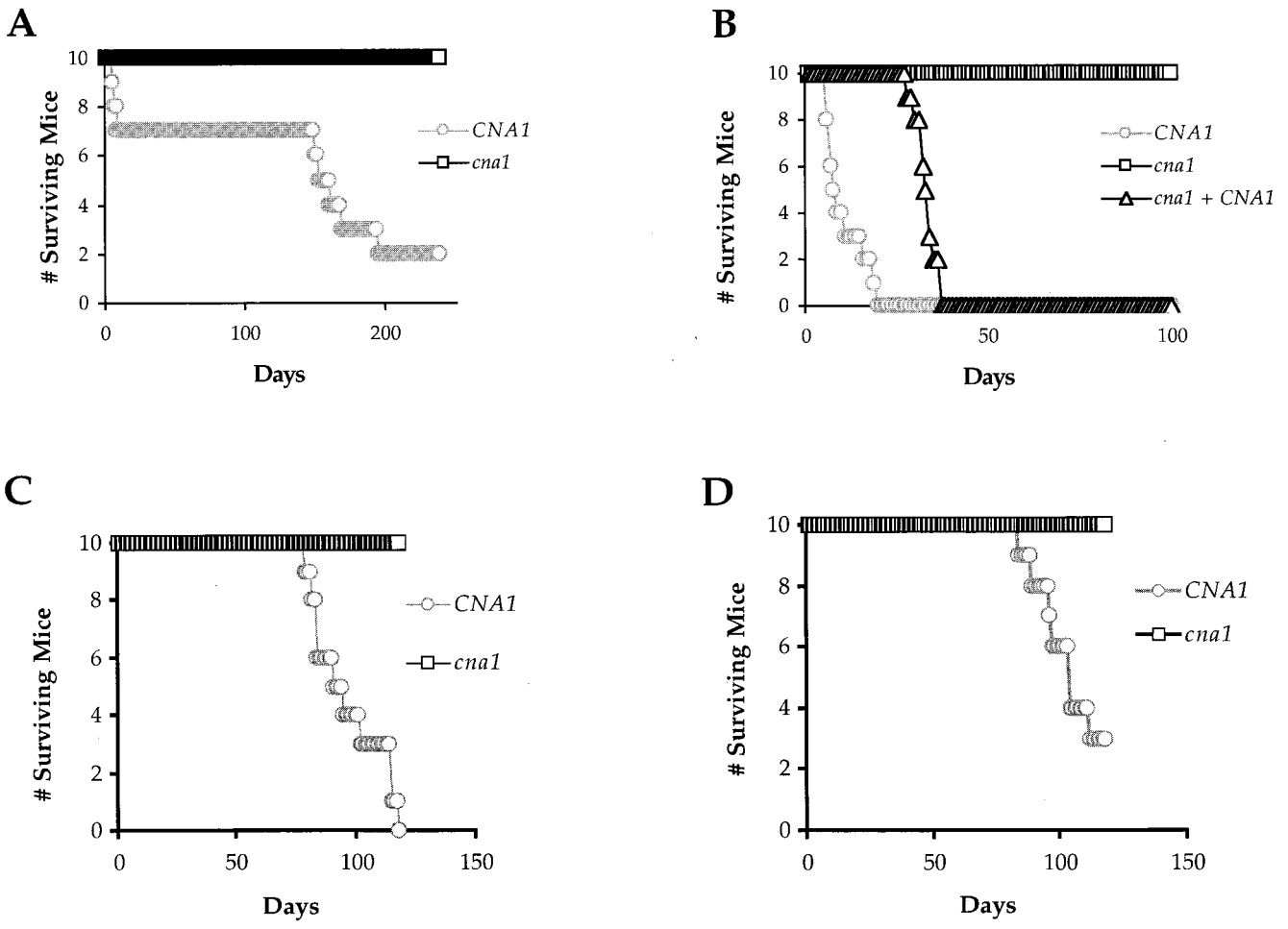

FIG. 2. Calcineurin is required for virulence of *C. neoformans* serotype D and serotype A strains in mice. BALB/c mice (10 for each strain) received injections in the lateral tail vein of 10^7 cells of the prototrophic *CNA1* wild-type *MAT*α serotype D strain (JEC21) or the congenic *MAT*α *cna1* mutant strain (MCC2) lacking calcineurin A (A) or the isogenic serotype A *CNA1* wild-type (H99), *cna1* mutant (AO4), and *cna1* plus *CNA1* reconstituted (AO10) strains (B). C5-deficient mice (10 each) received injections in the lateral tail vein of 10^7 cells of prototrophic *CNA1* wild-type *MAT***a** serotype D strain (JEC20) or the isogenic *MAT***a** *cna1* mutant strain (MCC10) lacking calcineurin (C) or the prototrophic *CNA1* wild-type *MAT*α serotype D strain (JEC21) or the congenic MATα *cna1* mutant (MCC5) lacking calcineurin (D). Mice were observed twice daily and the number of mice surviving versus time were plotted.

virulence of both *MAT***a** and *MAT*α serotype D strains in both immunocompetent and immunodeficient mice.

Summary and conclusions. Serotype A calcineurin A mutants are hypersensitive to Na^+ and Li^+ compared to the isogenic wild-type strain (14). These findings suggest a role for calcineurin analogous to that in *S. cerevisiae*, where calcineurin controls expression of the Pmr2 ion pump that effluxes Na^+ and Li^+ (26). In contrast, in the *C. neoformans* serotype D strain, calcineurin does not appear to regulate cation homeostasis because calcineurin mutants were as sensitive to cations as were isogenic wild-type strains. In fact, the wild-type congenic serotype D strains were inherently more sensitive to cation stress than serotype A strain H99, indicating significant physiological differences between serotype A and serotype D strains under stress growth conditions. Our findings provide further support for the classification of serotype A and serotype D strains into distinct varieties of *C. neoformans*: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) (9).

Calcineurin is required for virulence of serotype D and serotype A strains in both rabbits and mice. Calcineurin is the target for the immunosuppressive antifungal agents CsA and FK506 and thus is an attractive target for antifungal agents (6, 7, 14, 15). The role of calcineurin in growth at 37°C and virulence in *C. neoformans* could not have been predicted from model organisms, because calcineurin is not required for growth at 37°C in *S. cerevisiae* and is only required for normal growth at 22°C but not at 37°C in *S. pombe* (28). Thus, studies of pathogens are necessary to establish the functions of specific genes in virulence.

Nucleotide sequence accession number. The nucleotide sequence of the serotype D calcineurin A *CNA1* gene has been deposited in the GenBank database under accession no. AF1559511.

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